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Enhancement of glucose isomerase activity by pretreatment with substrates prior to immobilization

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Abstract−To improve the activity of covalently immobilized glucose isomerase, the effects of glucose isomerase pretreatment with D-glucose or D-xylose prior to immobilization were investigated. Glucose isomerase was pretreated with D-glucose or D-xylose to prevent loss of activity, followed by immobilization on a silica gel surface. Pretreated immobilized glucose isomerase (PIGI) with 2.0 M D-xylose (194.0 U/g matrix) had higher activity than PIGIs with D-glucose. The optimal temperature, reaction time, and agitation speed for glucose isomerase pretreatment were 60 °C, 45 min, and 200 rpm, respectively. Consequently, the activity of PIGI with D-xylose was 254.9 U/g matrix, which is about 2.5 times higher than that of non-pretreated immobilized glucose isomerase (non-PIGI). PIGI also showed better reusability than non-PIGI, with 75.2% of its original activity being retained after 10 reuses.

Key words: Glucose Isomerase, Pretreatment, D-Xylose, Immobilization, Silica Gel

INTRODUCTION

D-Glucose/xylose isomerase (systematic name D-xylose ketol isomerase; EC 5.3.1.5), commonly referred to as glucose isomerase, is a very important enzyme in the food and soft drinks industry that catalyzes the conversion of D-glucose to D-fructose [1,2]. In particular, isomerization of glucose to fructose has commercial significance in the production of high-fructose corn syrup (HFCS), which is one of the most successful enzymatic processes [3,4]. Glucose isomerase has also received increased attention due to its potential application to ethanol production from hemicelluloses [5]. The production of rare monosaccharides, such as L-glucose, L-fructose, L-ribose, L-lyxose, D-allose, and L-galactose, by glucose isomerase has recently received much attention due to potential health and medical benefits [6-9]. However, the expense of glucose isomerase prevents it from being discarded after a single usage, and thus it should be immobilized for reuse and recovery from the reaction mixture. For these reasons, glucose isomerase is usually used by the food industry in an immobilized form [6,9].

Over the last decade, immobilized enzymes have primarily been used in the bio-processes for the production of food, pharmaceuticals, and other biologically-based fine products [10-13]. Though quite expensive, many enzymes used in the food industry can be immobilized on solid support, creating a biocatalyst that can be reused several times at minimal cost [14]. Physical adsorption and entrapment among the various methods for enzyme immobilization are the simplest methods, but they possess limitations such as desorption and enzyme leakage that could lower the stability and catalytic activity of the enzyme [10]. On the other hand, covalent bonding methods between enzyme and solid support have been widely studied due to the high bonding strength that is produced [12,13,15,16]. However, enzyme activity tends to decrease markedly due to immobilization of enzyme to the solid support by covalent bonding, which damages the active site and distorts the native conformation [10,13,17]. To address these problems, a pretreatment method was developed to prevent the activity loss during enzyme immobilization by covalent bonding [10,18,19].

The present study was performed to assess the pretreatment of glucose isomerase with D-glucose or D-xylose for the prevention of covalent bond formation near the active site during immobilization. To the best of our knowledge, this is the first study that has evaluated this method using glucose isomerase. This study aimed to further the understanding of pretreated immobilized glucose isomerase (PIGI) by determining the optimal pretreatment source and concentration, temperature, reaction time, and agitation speed. In addition, the reusability of PIGI was examined through repeated batch experiments.

MATERIALS AND METHODS

1. Materials

Glucose isomerase (EC 5.3.1.5) from *Streptomyces rubiginosus* was purchased from Hampton Research (CA, USA). D-Xylose was purchased from Junsei Chemical Co. (Tokyo, Japan), and D-glucose was provided by Daejung Chemicals and Metals Co. (Siheung, Korea). Silica gels were provided by Grace Davison Co. (Columbia, MD, USA). (3-aminopropyl)triethoxysilane (3-APTES) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Glutaraldehyde solution was purchased from Fluka (Buchs, Switzerland). All other chemicals used in this study were of reagent grade.

2. Preparation of Activated Silica Gels

One gram of silica gels was silanized with 20 mL of acetone solution containing 0.75 M 3-APTES at 50 °C for 2 h, washed with distilled water, and dried at 60 °C for 2 h. Glutaraldehyde was modified at 64 °C for 20 min, and then the dried silica gels were added to 20 mL of 0.21 M modified glutaraldehyde solution in 0.1 M so-

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dium phosphate buffer (pH 8.0) after it was incubated at 20 °C for 2 h [20]. The activated silica gels were washed with distilled water and dried at 60 °C for 2 h.

3. Pretreatment of Glucose Isomerase before Immobilization

Two different substrates, D-glucose and D-xylose, were added to 10 mL of glucose isomerase solution containing 20 mM $MgSO_4 \cdot 7H_2O$ and 1 mM $CoCl_2 \cdot 6H_2O$. Mixtures were incubated at various temperatures (50-80 °C) and agitation speeds (50-250 rpm) from 15 min to 120 min, after which the glucose isomerase solutions were used for immobilization as described below.

4. Immobilization of Glucose Isomerase

The activated silica gels were added to the glucose isomerase solution and reacted at 20 °C with constant stirring. The immobilized glucose isomerase (IGI) was washed with 0.2 M sodium phosphate buffer (pH 7.0) and then recovered by filtration. The surface of silica gel was analyzed by a field emission scanning electron microscope (FE-SEM, Hitachi S-4300, Japan) with and without the enzyme immobilization.

5. Assay of Immobilized Glucose Isomerase Activity

IGI activity was assayed by measuring the concentration of D-fructose converted from D-glucose under the described assay conditions. The reaction mixture contained 10 mL of 0.2 M sodium phosphate buffer at pH 7.0, along with 0.2 M glucose, 20 mM $MgSO_4 \cdot 7H_2O$, 1 mM $CoCl_2 \cdot 6H_2O$, and IGI. The enzyme reaction was conducted at 60 °C and 100 rpm for 30 min, after which 2 mL of 20% (v/v) HCl was added to the reaction mixture to stop the reaction. The concentration of D-fructose was then measured by the HCl-resorcinol method in which resorcinol forms a red-colored complex with D-fructose [5] whose absorbance was read at 520 nm. For reuse, IGI was recovered after a single batch reaction by centrifugation, washed with the same buffer, and then reused for the next batch reaction. One unit of glucose isomerase was defined as the amount of enzyme that catalyzed the conversion of 1 μmol of D-glucose to D-fructose per minute under the assay conditions described. The activity of IGI was expressed in U/g matrix unit; the relative activity was calculated as a percentage of the maximum activity.

6. Determination of Immobilized Protein

The amount of immobilized protein was determined from the difference between total protein and the amount remaining in the solution after immobilization. The protein concentration was determined using Bio-Rad protein assay reagent according to the manufacturer's protocols (Bio-Rad Laboratories, Hercules, USA), and the absorbance was measured at 750 nm.

RESULTS AND DISCUSSION

1. Effects of Pretreatment of Glucose Isomerase with D-Glucose

To investigate the effects of glucose isomerase pretreatment, D-glucose (2.0 M) was added to the glucose isomerase solution prior to immobilization. For enzyme immobilization, the activated silica gels were added to the non-pretreated as well as pretreated glucose isomerase solutions, and glucose isomerases were subsequently immobilized on activated silica gels for two different time periods (12 and 24 h). FE-SEM analysis was carried out to confirm the change of silica gel surface. Fig. 1 shows SEM images of silica gel surface before (Fig. 1(a)) and after the enzyme immobilization (Fig. 1(b) and (c)). When glucose isomerase was immobilized, the roughness of silica gel surface decreased significantly, compared with that without enzyme immobilization. This result indicates that glucose iso-

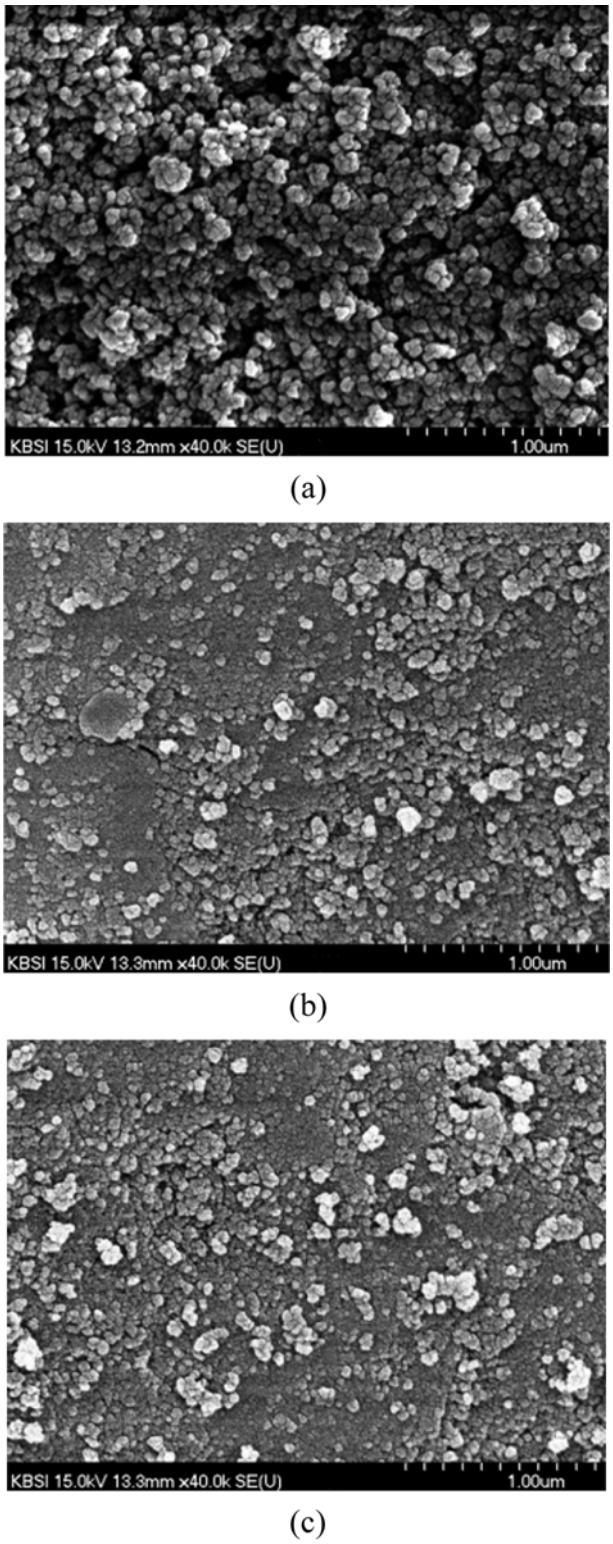

Fig. 1. FE-SEM images of silica gel surface (a) before immobilization, (b) after immobilization of non-pretreated glucose isomerase, and (c) after immobilization of pretreated glucose isomerase.

Table 1. Effects of pretreatment* on the IGI activity and amount of immobilized protein

Trials	Immobilization time (h)	Glucose isomerase	Immobilized protein (mg/g matrix)	Activity (U/g matrix)	Increased activity (%)
1	12	Non-pretreated	0.23	102.4	51.2
		Pretreated	0.25	154.8	
2	24	Non-pretreated	0.29	89.9	45.3
		Pretreated	0.32	130.6	

*The glucose isomerase was pretreated with 2.0 M D-glucose in 10 mL of 0.2 M sodium phosphate buffer (pH 7.0, containing 20 mM $MgSO_4 \cdot 7H_2O$ and 1 mM $CoCl_2 \cdot 6H_2O$) at 60 °C and 150 rpm for 30 min. The data shown are the mean of two independent experiments

merase was successfully immobilized on a silica gel surface. Table 1 shows the amounts of immobilized protein and the activity of PIGI and non-pretreated immobilized glucose isomerase (non-PIGI). The amounts of protein immobilized by the pretreated glucose isomerases (0.25 and 0.32 mg/g matrix) were only slightly higher than the amounts immobilized by the non-pretreated glucose isomerase (0.23 and 0.29 mg/g matrix) after 12 and 24 h of immobilization, respectively. However, we found that the activity of IGI was strongly dependent upon pretreatment with D-glucose prior to immobilization. Table 1 shows that the activity of PIGIs was 154.8 and 130.6 U/g matrix after immobilization for 12 and 24 h, respectively. These values are 51.2% and 45.3% higher than those of non-PIGIs (102.4 and 89.9 U/g matrix), respectively. These findings indicate that pretreatment of glucose isomerase with D-glucose before immobilization effectively improved the immobilized enzyme activity. Our previous studies showed that activity of immobilized lipase and β-galactosidase are strongly improved by a simple pretreatment method using soybean oil and lactose, respectively [10,18,19]. This result suggests that the effect of enzyme pretreatment prior to immobilization was due to steric interference at the enzyme active site by the substrate, and that the enzymatic regions far from the active site reacted with the silica gel surface [10]. Our results using glucose isomerase are similar to results obtained in previous studies using lipase and β-galactosidase [10,18,19]. Improved IGI activity by pretreatment with D-glucose is due to a large conformational change caused by a hydride shift that occurs upon the highly-ordered binding of substrate to the active site via Mg^{2+} and Co^{2+} [9]. Accordingly, Mg^{2+} and Co^{2+} with the substrate play an important role in enhancing the activity of IGI. This indicates that this pretreatment method induced steric hindrance in the vicinity of the active site that prevented covalent bond formation during immobilization [10]. In turn, this resulted in reaction of enzymatic regions far from the active site with the activated solid support, thus maintaining IGI activity at a high level. We also consider the pretreatment method to be useful for improving the activity of enzymes covalently immobilized on solid surfaces. In particular, when non-pretreated and pretreated glucose isomerases were immobilized for 24 h, the activity of non-PIGI and PIGI decreased by 12.2% and 15.6%, compared to that of immobilized glucose isomerases for 12 h, respectively. Therefore, immobilization in all subsequent experiments was carried out for 12 h.

2. Effects of Sugar Source and Concentration on Pretreatment

The main natural substrates of glucose isomerases are D-xylose and D-glucose. To gain deeper insight into glucose isomerase pretreatment, the effects of substrate source (D-xylose and D-glucose) were investigated at different concentrations (0.5-3.0 M). Fig. 2 shows

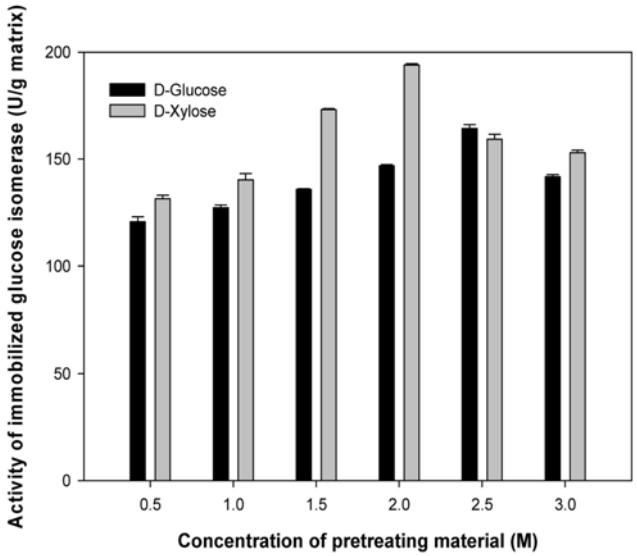

Fig. 2. Effects of sugar source and concentration on the pretreatment of glucose isomerase. The pretreatment of glucose isomerase was conducted in 10 mL of 0.2 M sodium phosphate buffer (pH 7.0, containing 20 mM $MgSO_4 \cdot 7H_2O$ and 1 mM $CoCl_2 \cdot 6H_2O$) at 60 °C and 150 rpm for 30 min. The data shown are the mean of two independent experiments.

the activity of PIGIs with different concentrations of D-xylose or D-glucose. It was found that the activity of PIGIs was higher compared to non-PIGI (Table 1 and Fig. 2). Specifically, the activity of PIGIs with D-xylose or D-glucose increased proportionally up to concentrations of 2.0 M or 2.5 M, respectively, after which it started to decrease. However, D-xylose as the substrate for pretreatment showed a more remarkable effect compared to D-glucose. The considerable activity (194.0 U/g matrix) was obtained with 2.0 M D-xylose, followed by 1.5 M D-xylose. In contrast, PIGIs with various concentrations of D-glucose showed comparatively lower activity compared to PIGIs with D-xylose. For example, the activity of PIGI with 2.5 M D-glucose was 164.2 U/g matrix, a value 15.4% lower than that of PIGI with 2.0 M D-xylose. We believe that the difference in D-xylose and D-glucose as enzyme pretreatments was due to different K_m and V_{max} values as well as formation of an isomer and C-2 epimer by glucose isomerase. Glucose isomerase mostly displayed higher affinity for D-xylose than for D-glucose. The K_m value of glucose isomerase for D-xylose was over 10 times smaller than that for D-glucose [3,6]. Moreover, the isomerization rate of D-xylose by *S. rubiginosus* glucose isomerase was considerably faster

than that of D-glucose [9,21]. Due to a high affinity and reaction rate, pretreatment of glucose isomerase with D-xylose would be more effective in preventing covalent bond formation near the active site during immobilization. On the other hand, several researchers previously reported that glucose isomerase from *S. rubiginosus* catalyzes the C-2 epimerization of D-xylose and D-glucose, although the latter reactions are very slow [6-9]. In this case, glucose isomerase catalyzed reactions through which two different products from each substrate were formed. Specifically, glucose isomerase with D-xylose and D-glucose produced D-xylulose/D-lyxose and D-fructose/D-mannose, respectively. These sugars (isomers and C-2 epimers) have different affinities for glucose isomerase. It is therefore believed that the isomer and C-2 epimer of D-xylose influences the pretreatment of glucose isomerase.

3. Effect of Temperature, Reaction Time and Agitation Speed on Pretreatment

The effect of temperature on the pretreatment of glucose isomerase was investigated at temperatures ranging from 50 °C to 80 °C with 2.0 M D-xylose. As shown in Fig. 3(a), the activity of PIGIs was increased as the temperature increased, with a maximum value at 60 °C. Compared to 60 °C, the activity was decreased by 9.7% and 6.4% at 55 °C and 65 °C, respectively, but more rapidly at 75 °C and 80 °C. Pastinen et al. [9] reported that the reaction rate of *S. rubiginosus* glucose isomerase increased up to 70 °C. In this case, the activity of PIGI did not increase above 60 °C. However, the ratio of substrate/isomer/C-2 epimer shifted as a function of temperature [1,8,9]. The amount of isomer and C-2 epimer was increased at high temperature while the amount of substrate was decreased. We therefore suggest that the amount of D-xylulose and D-lyxose along with that of isomer and C-2 epimer of D-xylose plays an important role in the pretreatment of glucose isomerase. In addition, the loss of activity was probably due to enzyme denaturation at elevated temperature. It is further noteworthy that *S. rubiginosus* glucose isomerase is a tetramer composed of four identical polypeptides with molecular weights of 43 kDa [1]. Therefore, thermal stabilization of immobilized glucose isomerase presumably requires effective attachment of four monomers.

The effect of time on glucose isomerase pretreatment was also examined from 15 min to 120 min. Fig. 3(b) shows the activity of IGIs pretreated with D-xylose for different time periods. The optimal pretreatment time was 45 min, but the activity was decreased by 9.4% and 7.4% after pretreatment for 30 and 60 min, respectively. When pretreatments were conducted for longer than 45 min, activity of PIGIs remarkably decreased. Especially, pretreatment of glucose isomerase with D-xylose for 120 min resulted in a 53.6% reduction in the activity. These results indicate that the time of glucose isomerase pretreatment strongly influenced PIGI activity. The industrial production of HFCS is usually carried out at temperatures below 60 °C in order to prolong half-life and reduction of thermal inactivation of glucose isomerase [2,22]. In this study, increased reaction time of glucose isomerase with D-xylose at 60 °C may have caused denaturation and loss of enzyme activity.

Glucose isomerase solutions were pretreated with agitation at various speeds from 50 rpm to 250 rpm. The activity of PIGIs was increased in proportion to agitation speed up to 200 rpm, above which it did not increase any further (Fig. 3(c)). Mixing was attempted to increase mass transfer. Our results indicate that mass transfer was

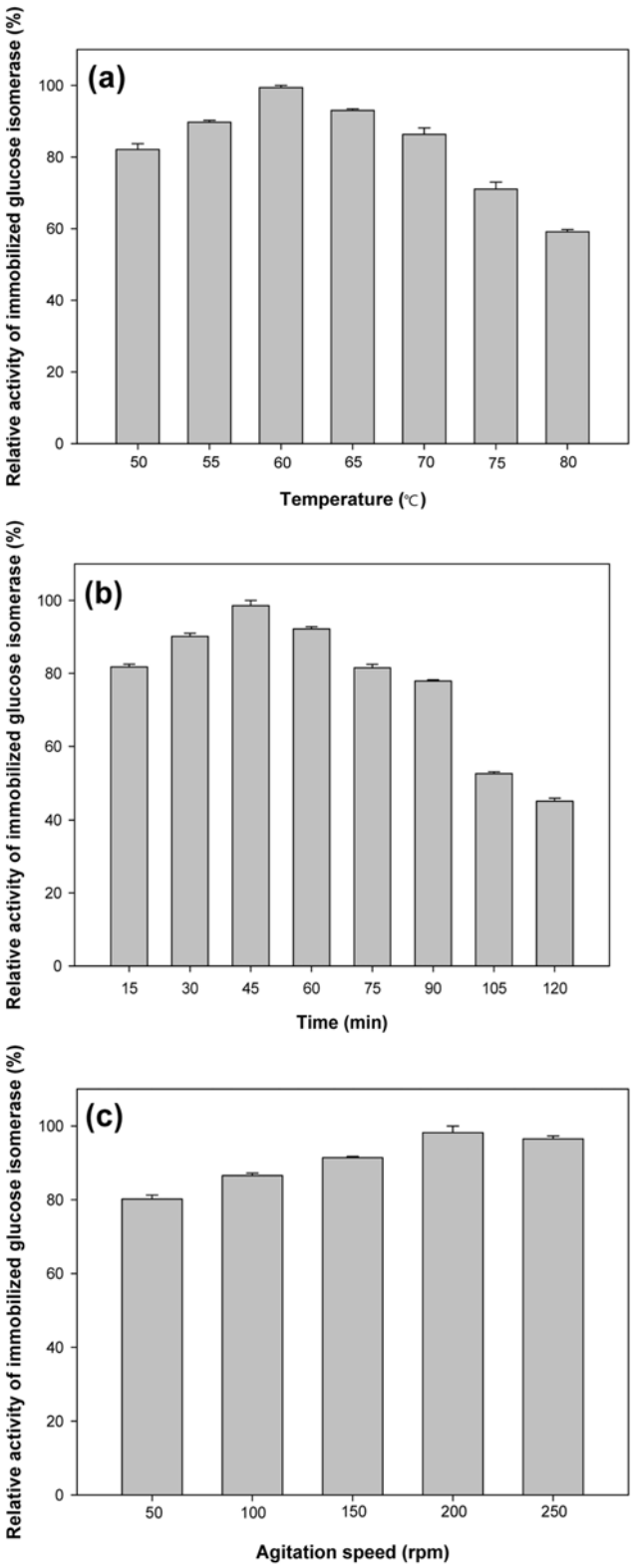

Fig. 3. Effects of temperature, reaction time and agitation speed on the pretreatment of glucose isomerase. The pretreatment of glucose isomerase was conducted with 2.0 M D-xylose in 10 mL of 0.2 M sodium phosphate buffer (pH 7.0, containing 20 mM $MgSO_4 \cdot 7H_2O$ and 1 mM $CoCl_2 \cdot 6H_2O$) (a) at 150 rpm for 30 min, (b) at 60 °C and 150 rpm, and (c) at 60 °C for 45 min. The data shown are the mean of two independent experiments.

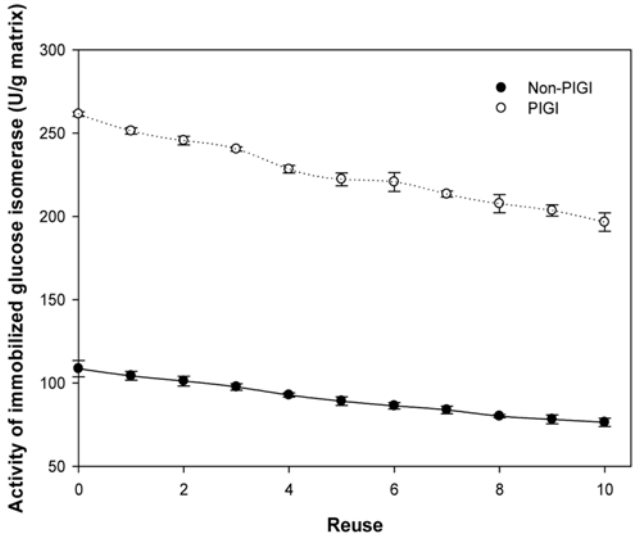

Fig. 4. Reusability of non-PIGI and PIGI. The glucose isomerase was pretreated with 2.0 M D-xylose in 10 mL of 0.2 M sodium phosphate buffer (pH 7.0, containing 20 mM $MgSO_4 \cdot 7H_2O$ and 1 mM $CoCl_2 \cdot 6H_2O$) at 60 °C and 200 rpm for 45 min. The concentrations of the IGIs were 10 mg/mL. The data shown are the mean of two independent experiments.

not improved at agitation speeds over 200 rpm. Therefore, the effect of agitation speed on the pretreatment of glucose isomerase was greatest at 200 rpm, resulting in PIGI activity of 254.9 U/g matrix. Especially, it was observed that the effect of agitation speed was less sensitive compared to other factors.

4. Reusability of Immobilized Glucose Isomerase

The reusability of immobilized enzyme is extremely important for industrial applications [10]. To assess reusability, non-PIGI and PIGI were used for consecutive batches of isomerization with D-glucose. The activity of reused non-PIGI and PIGI is shown in Fig. 4, with the first batch showing activity of 108.7 and 261.4 U/g matrix, respectively. The activity of both IGIs decreased as the number of reuses was increased. PIGI showed better reusability than non-PIGI. After 10 reuses, the activity of non-PIGI and PIGI was 76.4 and 196.6 U/g matrix, respectively. Especially, PIGI retained 75.2% activity after 10 reuses. Although the activity of PIGI decreased in response to enzyme deactivation or loss, our immobilization method undoubtedly led to increased enzyme reusability.

CONCLUSION

This study evaluated the activity of IGI pretreated with D-glucose or D-xylose prior to immobilization. Pretreatment of glucose isomerase with D-xylose greatly improved enzyme activity after immobilization. The results of this study show that pretreatment of the enzyme with natural substrates combined with optimization of reaction conditions can remarkably improve the activity of immobilized enzyme. The concept of enzyme immobilization using pretreatment is a relatively new one. In particular, the immobilization of glucose isomerase has to our knowledge never been published. Although the activity of many enzymes is reduced by immobilization using covalent bond, this study found that it may be possible to recover enzyme activity that has been decreased due to damage of the active site. Moreover, the immobilization method demonstrated in this study for the improvement of immobilized enzyme activity is much simpler than genetic and protein engineering techniques. The role of substrate/isomer/C-2 epimer in mechanistic interpretations of glucose isomerase pretreatment is still not clear. Therefore, further studies should explain the effect of substrate/isomer/C-2 epimer on glucose isomerase pretreatment.

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REFERENCES

1. M. A. Borgi, K. Srih-Belguith, M. Ben Ali, M. Mezghani, S. Tranier, R. Haser and S. Bejar, *Biochim.*, **86**, 561 (2004).
2. B. S. Hartley, N. Hanlon, R. J. Jackson and M. Rangarajan, *Biochim. Biophys. Acta*, **1543**, 294 (2000).
3. S. H. Bhosale, M. B. Rao and V. V. Deshpande, *Microb. Rev.*, **60**, 280 (1996).
4. M. Dadvar and M. Sahimi, *Chem. Eng. Sci*., **57**, 939 (2002).
5. S. S. Tükel and D. Alagöz, *Food Chem*., **111**, 658 (2008).
6. J. Jokela, O. Pastinen and M. Leisola, *Enzyme Microb. Technol*., **31**, 67 (2002).
7. K. Leang, G. Takada, Y. Fukai, K. Morimoto, T. B. Granström and K. Izumori, *Biochim. Biophys. Acta*, **1674**, 68 (2004).
8. A. Vuolanto, O. Pastinen, H. E. Schoemaker and M. Leisola, *Biocatal. Biotransform*., **20**, 235 (2002).
9. O. Pastinen, K. Visuri, H. E. Schoemaker and M. Leisola, *Enzyme Microb. Technol*., **25**, 695 (1999).
10. Y. S. Song, J. H. Lee, S. W. Kang and S. W. Kim, *Food Chem*., **123**, 1 (2010).
11. L. L. Zhao, J. Pan and J. H. Xu, *Biotechnol. Bioprocess Eng*., **15**, 199 (2010).
12. C. Zhou, A. Wang, Z. Du, S. Zhu and S. Shen, *Korean J. Chem. Eng*., **26**, 1065 (2009).
13. X. S. Zhao, X. Y. Bao, W. Guo and F. Y. Lee, *Mater. Today*, **9**, 32 (2006).
14. P. S. J. Cheetham, *Handbook of enzyme biotechnology*, Ellis Horwood, Frome (1985).
15. H. W. Wang, I. H. Kim, C. S. Park and J. H. Lee, *Korean J. Chem. Eng*., **25**, 801 (2008).
16. J. Y. Lee, H. Y. Shin, S. W. Kang, C. Park, K. K. Oh and S. W. Kim, *Biotechnol. Bioprocess Eng*., **14**, 687 (2009).
17. C. Giacomini, A. Villarino, L. Franco-Fraguas and F. Batista-Viera, *J. Mol. Catal. B-Enzym*., **4**, 313 (1998).
18. D. H. Lee, J. M. Kim, S. W. Kang, J. W. Lee and S. W. Kim, *Biotechnol. Lett*., **28**, 1965 (2006).
19. D. H. Lee, J. M. Kim, H. Y. Shin and S. W. Kim, *J. Microbiol. Biotechnol*., **17**, 650 (2007).
20. S. W. Park, Y. I. Kim, K. H. Chung and S. W. Kim, *Process Biochem*., **37**, 153 (2001).
21. O. Pastinen, H. E.Schoemaker and M. Leisola, *Biocatal. Biotransform*., **17**, 393 (1999).
22. Y. Ge, Y. Wang, H. Zhou, S. Wang, Y. Tong and W. Li, *J. Biotechnol*., **67**, 33 (1999).